LINOLEATE- and LINOLENATE-LIPOXYGENASE MUTANTS

The present invention concerns a process for the manufacture of plant lipoxygenases with altered positional specificity, together with the lipoxygenase produced by the procedure and its use in the hydroperoxylation of substrates.

The LOXs (linolenic acid: oxygen oxidoreductase; EC.1.13.11.12; LOXs) are widely distributed in the plant and animal domains (Siedow, J.N. (1991) Ann. Rev. Plant Physiol. Plant Mol. Biol. 42, 145-188; Yamamoto S. (1992) Biochem. Biophys. Acta 1128, 117-131). These enzymes represent a family of iron-containing dioxygenases which catalyse a regional (or positional) and stereoselective oxygenation of polyunsaturated fatty acids to hydroperoxy-derivatives (Rosahl, S. (1996) Z. Naturforsch. 51c, 123-138). In mammals LOXs are classified according to their specificity for particular positions in the oxygenation of arachidonic acid (Yamamoto, S. (1992) Biochem. Biophys. Acta 1128, 117-131; Schewe, T., Rapaport, S. M. & Kühn, H. (1986) Adv. Enzymol. Mol. Biol. 58, 191-272). Since arachidonic acid is not present in higher plants, or only in limited quantities as a component of stored lipids, LOXs from plants are classified as 9- and 13-LOXs. This nomenclature derives from the position in the linoleic acid (LA) at which the oxygenation takes place (Gardner, H.W. (1991) Biochem. Biophys. Acta 1084, 221-239). Lately a comprehensive classification of plant LOXs on the basis of a comparison of primary structures has been proposed (Shibata, D. & Axelrod, B. (1995) J. Lipid Mediators Cell Signal. 12, 213-228). The specificity of a LOX for a particular position is the result of two catalytic partial reactions:

(i)

of the regional and stereospecific removal of hydrogen, by which, in fatty acids containing several double bonds (such as linolenic acid, arachidonic acid or icosapentic acid) hydrogen removal in various positions can result.

(ii)

of the regional and stereospecific insertion of oxygen (by which the oxygen can be inserted in various positions (the +2 and -2 position) (compare Figure 1). In that way a fatty acid with 3 double-allylic methylenes, such as arachidonic acid can be oxygenated from a LOX to 6 regioisomeric hydroperoxy derivatives (HPETEs), that is, to 15- and 11-HPETE (these arise from the removal of hydrogen at position C-13), 12- and 8-HPETE (these arise from the removal of hydrogen at position C-10) and 9- and 5-HPETE (these arise from the removal of hydrogen at position C-7). Experiments with 12- and 15-LOX from mammals show that the position of the hydrogen removal can be altered if critical amino-acids are changed through directed mutagenesis (Borngraber S., Kuban R.J, Anton, M. & Kühn H. (1996) J. Mol. Biol. 264, 1145-1153; Sloane, D.L. Leung, R., Craik, C.S. & Sigal, E. (1991) Nature 354, 149-152). Attempts to change the LOX reactivity from a +2 to a -2 rearranging from or *vice versa* (e.g. converting a linoleate 13-LOX to a 9-LOX) with the assistance of directed mutagenesis have so far not been successful.

The technical problem underlying the present invention was to provide a method by which the required positional specificity of the LOX could be made available.

This problem is solved according to the invention through a process by which one or more amino-acids undergo substitution in a wild-type LOX.

Figure 1 shows the specificity of a LOX reaction with substrates containing two allylic methylenes.

Figure 2 shows the direct and inverse orientation of the substrate in the active centre of the LOX.

Figure 3 shows a model of the enzyme substrate interaction of the wild-type LOX from cucumber and of the mutant H608V (corresponding to H597V, where in the latter nomenclature the numeration according to the sequence from Figure 5 is used).

Figure 4 shows the HPLC analysis of hydroxylated fatty acids procured with the aid of wild-type LOX from cucumber and of the H597V mutant from LA.

Figure 5 shows the sequence of wild-type LOX from *Cucumis sativus*.

Figure 6 shows the HPLC analysis of hydroxylated fatty acids obtained with the aid of the mutant V531F from γ-linolenic acid.

Figure 7 shows the HPLC analysis of oxidated trilinolein, formed by wild-type enzyme and H597V mutants.

In a preferred embodiment the exchange of the amino-acids takes place in the region of the amino-acid position 527 to 536 or 593 to 602 of the LOX from *Cucumis sativus* or a corresponding position in a LOX from a different plant species. The amino-acid positions given above refer to the sequence under the access in number X92890 in the NIH data bank „Entrez“ or the sequence according to Figure 5. The positions in other LOXs corresponding to the amino-acid positions 527 to 536 or 593 to 602 of the lipoxygenase from *Cucumis sativus* in LOXs from other plant species can easily be determined by sequence comparisons between the sequence X92890 and the further protein sequences such as from soya beans, potatoes, *Arabidopsis*, tobacco or barley. The following Table 1 shows the result of an amino-acid comparison between the enzyme originating from cucumber and the corresponding positions in the enzymes from other plants. The first group (13-LOX) shows a comparison between LOXs which insert a hydroperoxy group at position 13, while the second group (9-LOX) shows a comparison between groups which insert a hydroperoxy residue at position 9.

Table 1

Comparison of amino-acid residues which are presumably involved in the specificity of a plant LOX for a particular position (13 and/or 9).

ENZYME residue	Access no.	Position of the amino-acid residue	Amino-acid
13-LOX			
Cucumber lipid body LOX	X92890	596/597	Thr/His
LOX-1 from soya beans	P08170	556/557	Thr/Phe
LOX-H1 from potatoes	X96405	614/615	Ser/Phe
LOX-2 from *Arabidopsis*	P38418	611/612	Cys/Phe
9-LOX			
LOX from potatoes	P37831	579/580	Thr/Val
Elicitor-induced LOX from tobacco	X84040	580/581	Thr/Val
LOX-A from barley grain	L35931	574/575	Thr/Val

The sequence at position 527 to 536 reads TVNDVGYHQL according to the single-letter code for amino-acids in the deposited sequence X92890. The sequence at position 593 to 602 reads IETTHYPSKY (sequence according to X92890).

In an especially preferred embodiment the substitution at position 531 and/or 597 results in the sequence X92890. At position 531 in the wild type a Val- residue and at position 597 a His-residue are found.

In a further preferred embodiment the residue at position 531 is replaced by a Phe- or a His-residue and at position 597 by a Val- or a Phe- residue.

Most particularly preferred is an embodiment in which the substitution at position 531 represents a Val -> Phe and at position 597 a His -> Val substitution. Preferably in each case only one of the substitutions mentioned takes place in a given wild type. In that way the substitution in the region of amino-acid positions 527 to 536 leads to the conversion of the 13-LOX from the lipid bodies of *Cucumis sativus* to a γ-linolenic acid 6-LOX, while the substitution at position 597 leads to conversion of the linolenic acid 13-LOX to a linolenic acid 9-LOX. Subsequently both these mutants are described as V531F and H597V. The wild-type sequence is shown as Figure 5. Positions 531 and 597 are indicated.

Preferably the exchange of amino-acids is performed in the wild-type with the aid of directed mutagenesis, as is known in the state of the art (cf. e.g. Feussner, I., Bachmann, A., Höhne, M.& Kindl, H. (1998) FEBS Lett. 431, 433-436).

The present invention concerns in addition LOX mutants which are obtainable by the above-mentioned process.V531F and H597V are the preferred mutants, as explained in more detail above. The LOXs according to the invention are manufactured with the aid of known state-of-the-art procedures such as directed mutagenesis and the associated protein expression.

Furthermore the present invention concerns nucleic acids coding for the LOXs according to the invention. Proceeding from the state-of-the-art available wild-type sequences, the sequences according to the invention can be constructed though directed mutagenesis.

The present invention furthermore concerns vectors into which the nucleic acids according to the invention are introduced with the aim of cloning and expression. Corresponding cloning and expression vectors are adequately known to the skilled person from the present state of the art (cf. Maniatis et al. Molecular Cloning: a Laboratory Manual (1989), Cold Spring Harbor Laboratory Press).

The present invention concerns in addition a cell into which the nucleic acid according to the invention or the vector according to the invention is inserted. Following insertion of the nucleic acid or the vector the cell is then in a position to express a LOX for the first time or in an increased quantity. In this way the fatty acid pattern of a cell can be specifically altered, with the result that the phenotype of the cell can be altered in various respects. Among other things this may include a different composition of the cell membrane.

Finally, from the abovenamed cells new plants or plant parts can be generated through *in vitro* cultivation procedures. For the construction of such transgenic plants the well-known transformation system based on the *Agrobacteria* and Ti-plasmid derivatives can for example be used.

The LOXs according to the invention permit for the first time the manufacture of new γ-linolenic acid derivatives in large quantity. For this, γ-linolenic acid as substrate is incubated under suitable conditions with the LOXs according to the invention. Depending on the inserted LOX mutant, hydroperoxylation of the γ-linolenic acid then preferably occurs at position 6 or position 9 or positions 6 and 9.

Especially preferred is a γ-linolenic acid derivative containing a hydroperoxy group at position 6. The derivative can then be converted easily into the hydroxy derivative.

Such a γ-linolenic acid derivative has not been accessible hitherto, since it lacked a LOX with suitable position specificity.

Further examples serve as illustration of the invention.

1. Manufacturing the mutant H597V

Materials:

The chemicals used were drawn from the following sources: the standards for chiral and racemic hydroxy fatty acids came from Chayman Chem (Ann Arbor, Mi, USA) and trilinolein (TL) from Sigma, Deisenhofen (Germany). Methanol, hexane, 2-propanol (all of HPLC grade) came from Baker (Griesheim, Germany). Restriction enzymes were obtained from New England Biolabs (Schwalbach, Germany).

Directed mutagenesis and protein expression:

For the bacterial expression of wild-type LOX and for directed mutagenesis the plasmid pQE-30 (Qiagen, Hilden, Germany), which contained the cDNA of the LOX from lipid bodies of cucumber cotyledons as an insert (LOXpQE 30: cf. Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436) was used. The mutagenesis was carried out with the aid of the QuikChange Mutagenesis Kit from Stratagene (Heidelberg, Germany). Oligonucleotides with the appropriate base exchanges were obtained from MWG-Biotech (Ebersberg, Germany). For the analysis of the mutations further conservative base exchange was initiated in order to create new restriction division sites or to delete existing ones. In addition all the mutations were sequenced and at least three different bacterial clones expressed and used for the investigation of enzymatic properties. The expression of LOXpQE-30 and all the mutations was carried out according to Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436. Cells from 1-litre cultures were resuspended in 5 to 7ml lysis buffer and with the aid of an ultrasound needle broken up with pulses each of 30 seconds, and the cell debris made into pellets. The affinity purification of the polyHis-extended LOX was carried out as previously described (cf. Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436).

Activity assay and preparation of samples:

For product analysis 0.9ml of the cell lysate was incubated at room temperature for 30 minutes with 0.9mM LA, 0.9mM γ-linolenic acid or 1.2 mM trilinolein (end concentration) in 100mM tris buffer pH 7.5. The reactions were stopped by the addition of sodium borhydride, to convert the hydroperoxy fatty acids to the corresponding hydroxy compounds. The tests were acidified to pH 3 and the lipids were extracted (cf. Bligh, E.G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917). The lower chloroform phase was recovered and the solvent medium steamed off. The remaining lipid was dissolved in 0.1ml methanol and aliquots subjected to HPLC analysis. For the alkaline hydrolysis of triacyl glycerine the lipid extracts were diluted with 0.4ml methanol. Eighty µl of 40% (w/v) KOH was added and the samples were incubated for 20 minutes at 60°C under an argon atmosphere. After cooling off to room temperature the tests were acidified with glacial acetic acid and the aliquots analysed by RP-HPLC.

Analysis:

The HPLC analysis was carried out with a Hewlett Packard 1100 HPLC system coupled to a diode detector. The RP-HPLC of free fatty acid derivatives was carried out on a Nucleosil C-18 column (Macherey-Nagel, 250 x 4 mm, 5µm particle size) with a solvent system of methanol/water/acetic acid (85/15/0.1; v/v/v) and a flow rate of 1ml/min. The absorptions at 234nm (absorption of the conjugated double bond system of the hydroxy fatty acids) and at 210nm (polyunsaturated fatty acids) were correspondingly recorded. Triacyl glycerine, which contains oxygenated LA, was separated on a Nucleosil C-18 column (Macherey-Nagel, Düren, Germany; 250 x 4mm, 5µm particle size) using a binary gradient system. The system included as solution A:

methanol/water/acetic acid (90/10/0.1, v/v/v), and as solution B methanol/acetic acid (100/0.1, v/v), and the following gradient programme was run through: 10 minutes at 100% solution A, then over 20 minutes with a linear increase of solution B to 100% solution B, followed by an isocratic run of 50 minutes at 100% solution B. The absorption at 234nm was recorded. The direct phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (HP, Waldbronn, Germany; 250 x 4mm, 5µm particle size) with a solvent system of n-hexane/2 propanol/acetic acid (100/2/0.1 v/v/v) at a flow rate of 1ml/minute. The enantiomer composition of the hydroxy fatty acids was analysed with the aid of chiral-phases-HPLC on a Chiralcell-OD column (Daicel Chem. Industrie, sales department of Baker Chem., Deventer, The Netherlands; 250 x 4.6mm, 5µm particle size) with a solvent system of hexane/2-propanol/acetic acid (100/5/0.1, v/v/v) at a flow rate of 1ml/min. (cf Feussner, I., Balkenhohl, T.J., Porzel, A., Kühn, H. & Wasternack, C. (1997) J. Biol.Chem. 272, 21635-21641).

Modelling the enzyme/substrate exchange activity through alteration of the structure with the aid of directed mutagenesis:

The structure investigations carried out on numerous lipoxygenases from a variety of sources and own investigations established that position 597, the one His residue from the lipid bodies carried by the cucumber, could be an appropriate point of attack for altering the positional specificity of 13-LOX. Consequently the mutant H597V was constructed with the aid of directed mutagenesis. The wild-type and the mutant were over-expressed as polyHis-prolonged fusion protein purified on a nickel-sepharose column. As expected, the HPLC analysis of the oxygenated LA product with the wild-type enzyme produced 13-H(P)ODE as main product (cf. figure 4). For the mutant H597V, however, 9-H(P)ODE was identified as the main product. A further mutant was constructed, in which the His residue at position 597 has been replaced by a further amino-acid, in which the further amino-acid residue occupies a greater volume

than valine but a smaller volume than histidine. The mutant H597M was constructed. This mutant also showed a strong preference for the formation of 9-H(P)ODE. The kinetic characterization of the wild-type in accordance with 13-LOX and the 9-LOX mutant H597M showed that the mutation led to a greatly increased substrate affinity and a reduction in the reaction speed. For the wild-type enzyme a K_M of 114.9 µM and an LA turnover at V_{max} condition (substrate saturation) of 12 s^{-1} were determined (23 points were measured between 100µM and 250µM LA concentration). In contradistinction to that a V_{max} of 2 s^{-1} and a K_M of 1333.3µM were calculated for the H597M mutant (21 points were measured between 300µM and 1400µM LA concentration). These data indicate that the substrate binding could be vigorously hindered by the mutant, so that more substrate is necessary to reach V_{max}. A further mutation was investigated in which a mutant was produced with the polar threonine at position 596 being replaced by an isoleucine, which is smaller but does not contain a polar hydroxy group. This mutant was catalytically active (comparable with the wild-type enzyme) but happened to show a scattered position specificity.

Specificity of the reaction with trilinolein:

Earlier investigations of the substrate specificity with LOX from the lipid bodies of the cucumber indicated the capacity of the enzyme to oxygenate esterified polyunsaturated fatty acids (cf. Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436; Feussner, I., Balkenhohl, T.J., Porzel, A., Kühn, H. & Wasternack, C. (1997) J. Biol. Chem. 272, 21636-21642). Since triacyl glycerine contains no free carboxyl groups, no actual differences are expected if the pattern of the oxygenation products of the wild-type are compared with the 9-LOX mutants. In fact it is found that the wild-type enzyme and the 9-LOX mutants show trilinoleate-13-LOX activity. Nevertheless the rates of trilinolein oxygenation by the 9-LOX mutants were only 50% of the activity measured for the wild-type enzyme. Furthermore the trilinolein oxygenation by the mutated enzyme led in fact to triacyl glycerine variants in which an LA residue was oxygenated. By contrast, with the wild-type enzyme all 3 linoleic acid residues were oxygenated (cf. Figure 7).

2. Manufacturing LOX mutants:

The reagents and procedures used in the manufacture of these mutants were in fact those described above for the H597V mutant. In what follows some deviations from the abovenamed procedure, which were specially adapted for the manufacture of the V531F mutant, are explained.

Directed mutagenesis and protein expression

The initial cDNA and the mutagenesis kit were as described above. For the analysis of the mutation further conservative base exchanges were carried out in order to produce a new restriction site for *BstEll*. For the manufacture of the V531F mutation the following primer was used: GCT TAT GTA ACT GTT AAT GAT TTC GGT TAC CAT CAA CTT ATT AGT CAT TGG TTG CAT AC (coding strand) and GTA TGC AAC CAA TGA CTA ATA AGT TGA TGG TAA CCG AAA TCA TTA ACA GTT ACA TAA (complementary strand). In addition the mutant was sequenced and 3 different bacterial colonies were expressed and used for the enzymatic investigations. The expression of LOXpQE-30 was carried out as described previously. The further processing also continued as already described above. The analysis of the fatty acid derivatives produced (the one hydroperoxy group contained in position 6) also continued as described above. The result of the SP-HPLC analysis of the conversion of γ-linolenic acid with V531F is shown in Figure 6. The following Table 2 shows a comparison of the specificity of the wild-type (cslbLOX) with the mutant (cslbLOXV_{531}F).

Table 2

Comparison of the product specificity of cslbLOX and cslbLOX$_{531}$F with γ-linolenic acid

Enzyme	(13S, 11E, 9Z, 6Z)-18:2	(10S, 12Z, 8E, 6Z)-18:2	(9S, 12Z, 10E, 6Z)-18:2	(6S, 12Z, 9Z, 7E)-18:2
cslbLOX	80%	17%	3%	0%
cslbLOXV$_{531}$F	26%	14%	9%	51%

3. Description of the figures

Figure 1 shows that the positional specificity of the LOX reaction depends on the site of the splitting off of the hydrogen and on the orientation of the radical. The [+2]- order of the radical shows that the oxygen is transferred to the second carbon atom in the direction of the methyl terminal of the substrate, counted from the site of the hydrogen removal. [-2] shows the inverse orientation of the ordering of the radical.

Figure 2 shows the direct and inverse substrate orientation at the active site of the LOX (modified from Gardner, H.W. (1989) Biochem. Biophys. Acta 1001, 274-281).

Figure 3 shows a 3-dimensional model of the enzyme-substrate exchange operation. In the illustration on the left the wild-type enzyme is shown. Here the methyl terminal of the fatty acid substrate comes into contact with the side-chain H608. The charged residue R758 is screened by the residue H608. In the illustration on the right the mutant H608V (≡ H597V) is shown. In the inverse orientation the negatively charged carboxyl group of the substrate can form a salt bridge with the positively charged nitrogen of R758.

Figure 4 shows the HPLC analysis of fatty acids with the mutant H597V. Equal quantities of LOX protein are incubated with 0.9mM LA at room temperature for 30 minutes. Following reduction of the lipids with sodium borhydride the reaction mixture is acidified to pH 3 with acetic acid and the lipids extracted. The oxygenated fatty acid derivatives are isolated by means of RP-HPLC and the individual position isomers analysed with the aid of SP-HPLC. The proportions of S and R are analysed with the aid of CP-HPLC (inserted illustrations).

Figure 5 shows the amino-acid sequence of wild-type lipoxygenase from *Cucumis sativus*.

Figure 6 shows the HPLC analysis of the fatty acid pattern as obtained with the mutant V5321F and γ-linolenic acid.

Figure 7 shows the HPLC analysis of oxidized trilinolein as a result of conversion with the wild-type enzyme or the mutant H597V. Equal quantities of LOX protein were incubated with an emulsion from 1.2mM TL for 30 minutes. The lipids were reduced with sodium borhydride and the reaction mixture acidified to pH 3 with glacial acetic acid. Following extraction of the lipids the analysis continued by means of RP-HPLC. A representative chromatogram of this analysis is shown. The numbers identify the LOX reaction products obtained: 1 means a TL derivative containing an oxygenated fatty acid; 2 means a doubly oxygenated TL isomer, and 3 means a TL oxygenated three times. For analysis of the positional isomers of LA residues the free fatty acid derivatives were obtained by means of alkaline hydrolysis and subsequent RP-HPLC. The positional isomers of hydroxylinoleic acid (HODE) were represented as molar ratios as determined by means of SP-HPLC, as shown in the accompanying illustrations. Optical isomers were ascertained by means of CP-HPLC.

The abbreviations used are:

CP-HPLC	for	chiral phase HPLC;
RP-HPLC	for	reverse phase HPLC;
SP-HPLC	for	direct phase HPLC;
HPETE	for	hydroperoxyarachidonic acid
13-H(P)ODE	for	(13S, 9Z, 11E)-13-hydro(pero)xy-9,11- octadecylic acid;
9(HP)ODE	for	(9S, 10E, 12Z)-9-hydro(pro)xy-10,12-octadecylic acid;
LA	for	linoleic acid;
LOX	for	lipoxygenase;
TL	for	trilinolein.